

## **Molecular determination of extended spectrum B-lactamases (ESBLs) antibiotics resistance genes in *E.coli* isolated from diarrhea in cattle**

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### **Summary:**

None response to the treatment by an antibiotic called antibiotics resistance result from some genes called resistance genes .This mechanism is widespread in most of the bacteria, like *E.coli* . All of the extended resistance genes called (ESBLs) is a typical example for study of some genes that resistance beta-lactam antibiotic is subject of this research.

Fifty feces sample were collected from cattle suffering from diarrhea in alqaiyyah city were cultured on selective media for *E.coli* , then DNA was extracted from all *E.coli* isolates for antibiotic resistance gene detection by PCR ; The results of this study revealed the prevalence of B-lactamase gene four B-lactamases genes in *E.coli* *blaAmpc* gene were (91.4%), the *bla<sub>ctx-m</sub>* gene were (80%), *bla<sub>Tem</sub>* were (62.8%) and finally and *blaSHV* gene were (22%) among isolates *E.coli* ; *bla<sub>AMPC</sub>* gene has high prevalence than others genes while *bla<sub>SHV</sub>* was a lower percentage than other genes .

### **Introduction:**

Diarrhea is the syndromes characterized by loose of fluids from the body; usually, lasts days will be developed to the dehydration case due to decrease the fluid less than normal level. The causative agent of the diarrhea is inflammation in the intestines due to a viral, bacterial, or parasite; these infections are often transmitted through contaminated food or contaminated water by the stool, or transmitted directly between the animals. *E.coli* considered most common in a family of Enterobacteriaceae which causes diarrhea in all age and all animals' types and in all the degree (1).most *Escherichia coli* strains are commensal microbial in the mammalian gastrointestinal gut, yet some strains can cause severe diarrhea illnesses in animals (2) and (3). *Escherichia coli* is the most opportunistic bacteria that cause enteritis. Many of *E.coli* cases don't a response to treatment for causes related to have genetic materials that have (4).  $\beta$ -Lactam antibiotics have been used on the wide aspect to treat *E. coli* disease; however, treatment by this group become without good results in last period. The rate of antibiotic resistance is increasing and this antibiotic become without any cure and increasing every year in the world (5).

The antibiotic used for several cascade ago, percentage of  $\beta$ -lactam resistant in enterobacteria started an increasing. Some of the enterobacteria group is opportunistic pathogens and develop new resistance mechanisms and new genes in the genetic material can prevent the antibiotic to kill the bacteria and resist the antibiotic beta-lactam group called extended spectrum beta- lactamases (ESBLs) (6).

(ESBLs) have considered as a huge problem spread in the worldwide with little of treatment methods (7) ESBLs are defined as B-lactamases that confer bacterial

resistance to some of the antibiotics like penicillins, cephalosporins, and aztreonam by deactivation these antibiotics, and are inhibited by B-lactamase inhibitors (8) (9). The organism that producing  $\beta$ -lactamase enzymes is used as mechanism action of bacterial resistance. (ESBLs) Extended-spectrum  $\beta$ -lactamases formed bacteria have much antibiotic non-respond also placed this gene on the plasmid in the cytoplasm that carries the genes (10-13). This study was designed to detect several types of (ESBLs) are included (*bla<sub>shv</sub>*) gene, (*bla<sub>CTX</sub>*) gene, (*bla<sub>TEM</sub>*) gene and (*bla<sub>Amp</sub>*) gene also another studies (14-19). The aim of our research is knowledge of the prevalence of beta-lactam resistance genes in *E. coli* has taken from diarrhea cases in cattle.

## Methods and Materials:

### Sample collection:

Fifty Fecal samples were collected from cattle suffering from diarrhea from different fields in Diwanya province. The samples were placed in sterile (25) ml container, transferred into Microbiology of Laboratory College of Veterinary Medicine to bacterial isolation.

### Bacterial identification:

*Escherichia coli* was isolated from fecal samples by inoculation on blood agar and maconcky agar then EMB broth at 37°C overnight for primary enrichment culture and the bacterial growth were culture on Eosin methylene blue media (EMB) and blood agar at 37°C overnight for selective isolation of purity culture *Escherichia coli* isolates.

### Bacterial DNA extraction

DNA extraction by using commercial DNA extraction kit (Presto Mini-DNA Bacteria Kit. Geneaid Biotech Ltd. Korea). The extraction method was depending on the manufacturing instructions by using Gram-negative bacteria DNA Protocol extraction method by using (10 mg/ml) proteinase K buffer.

### Nanodrop:

The extracted DNA was estimated by nanodrop device at 260-280nm and was preformed it store at deep freezer until used in PCR method.

### PCR( Polymerase Chain Reaction):

PCR technique was used for detection of four genes in *E. coli* (*bla<sub>TEM</sub>*, *bla<sub>SHV</sub>*, *Bla<sub>CTX-M</sub>*, and *bla<sub>AMPC</sub>* genes) the detection was depending on specific primer.

The PCR primers was used in current study to detect the extended-spectrum  $\beta$ -lactamases including (*Bla<sub>CTX-M</sub>* gene, *bla<sub>SHV</sub>* gene, *bla<sub>TEM</sub>* gene, and *bla<sub>AMPC</sub>* gene) were designed by NCBI Gene sequence database. The primers have provided from Company of Bioneer, in Korea table (1).

**Table (1): PCR primers and their sequence.**

Primer of gene	Sequence	Amplicon
<b>Bla<sub>CTX-M</sub></b>	<b>3AGCGATAACGTGGCGATGAA5</b>	<b>247bp</b>
	<b>5TCATCCATGTCACCAGCTGC3</b>	
<b>Bla<sub>SHV</sub></b>	<b>3CCGCCATTACCATGAGCGAT5</b>	<b>410bp</b>
	<b>5AATCACCACAATGCGCTCTG3</b>	

<b>Bla<sub>TEM</sub></b>	<b>3GGTGCAVGAGTGGGTTACAT5</b>	<b>531bp</b>
	<b>5TGCAACTTTATCCGCCTCCA3</b>	
<b>Bla<sub>AMPC</sub></b>	<b>3AAACGACGCTCTGCACCTTA5</b>	<b>670bp</b>
	<b>5TGTA CTGCCTTACCTTCGCG3</b>	

**preparation of PCR master mix:**

The mix was prepared using (Accu-Power®PCR-PreMix-Kit) master mix reagent and done depend on company instructions as following table (2).

**Table (2): Protocol of PCR reaction mixture volume and concentration.**

<b>Master mix</b>	<b>Volume</b>
<b>DNA template (10ng/μl)</b>	<b>5μl</b>
<b>Forward primer</b>	<b>1.5 μl</b>
<b>Reverse primer</b>	<b>1.5 μl</b>
<b>PCR water</b>	<b>12 μl</b>
<b>Total volume</b>	<b>20 μl</b>

PCR mix revealed table (2) placed in AccuPower PCR -PreMix which contains all other PCR substances which required to the reaction such as (, dNTPs, NTAq DA polymerase, 10% PCR buffer). Next step all the PCR tubes treat with vortex centrifuge for vibration for 3 minutes and transferred into thermocycler (MyGene, Bioneer. Korea).

**PCR thermocycler conditions:**

**Table (3): PCR thermocycler conditions**

PCR step	Temp.	Time	repeat
<b>Initial Denaturation</b>	<b>95C</b>	<b>5min</b>	<b>1</b>
<b>Denaturation</b>	<b>95C</b>	<b>30sec.</b>	<b>30 cycle</b>
<b>Annealing</b>	<b>60C</b>	<b>30sec</b>	
<b>Extension</b>	<b>72C</b>	<b>1 min</b>	
<b>Final extension</b>	<b>72C</b>	<b>5min</b>	<b>1</b>
<b>Hold</b>	<b>4C</b>	<b>Forever</b>	<b>-</b>

**PCR product analysis:**

The PCR final products were examined by electrophoresis with (1%) agarose gel using 1X TBE buffer, and stained with ethidium bromide and investigation under UV transilluminator (80) volts for one hour.

**Results:**

Thirty five out of fifty isolates of were identified as *E.coli*, while the rest is negative 15(30%) by using EMB media (contain all nutrient element for grow *E.coli* like PH as in the intestine, salt, bile acid .... etc) as below table (4).

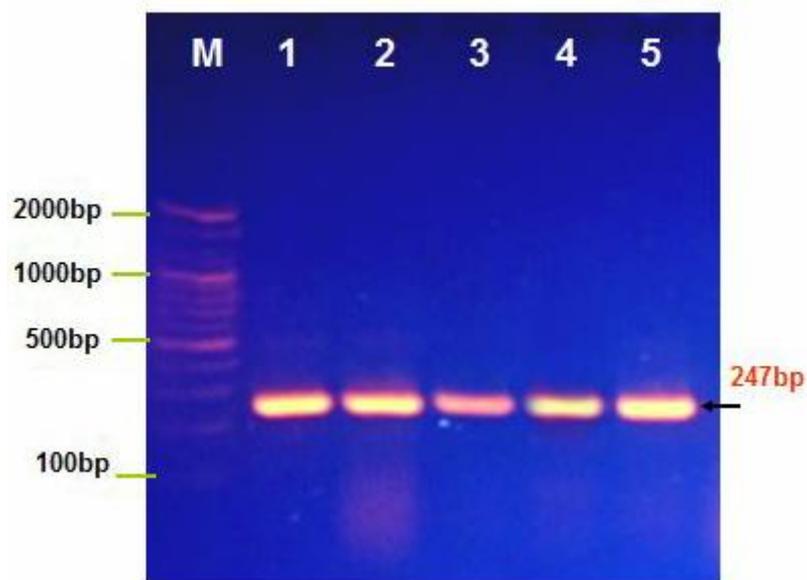
**Table (4): number and percent of *E.coli* isolated from 50 cattle feed samples**

Total isolates	Number <i>E.coli</i> isolates	Percent %	Number Of other bacteria	Percent %
<b>50</b>	<b>35</b>	<b>70%</b>	<b>15</b>	<b>30%</b>

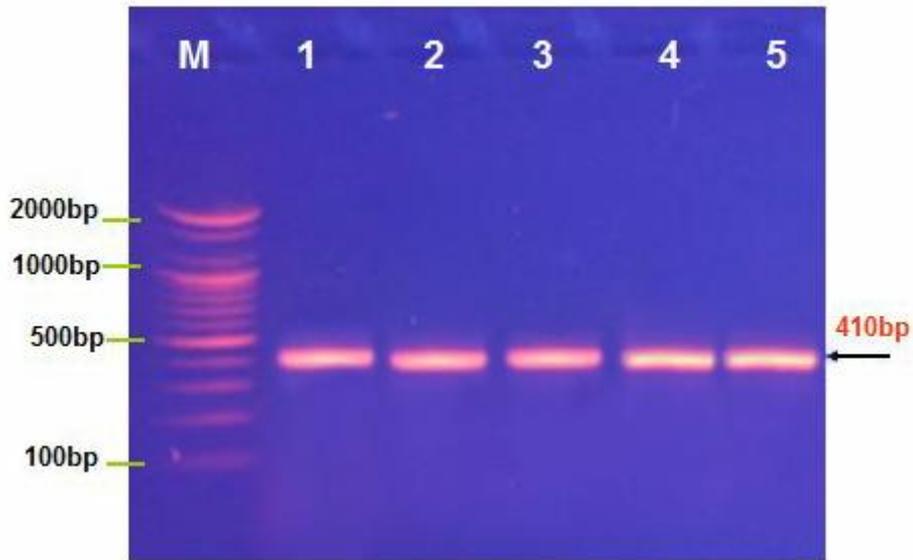
The Results of Polymerase chain reaction showed many of (ESBLs) (*bla<sub>A</sub>MPC*), (*Blactx-M*), (*bla<sub>TEM</sub>*), and (*bla<sub>SHV</sub>* genes) as following table (5); the figures (1) (2) (3) and (4). :

**Table(5): ESBLs gene name and the Percent mant genes were detected by PCR as. *blaAmpc*, *blactx-m*, *blaTem* and *blaSHV* genes**

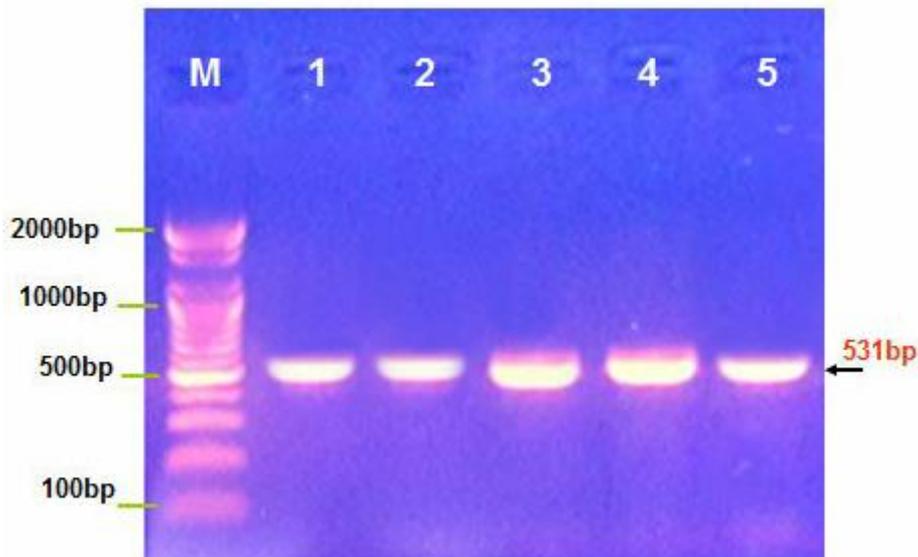
ESBLs gene name	Number of isolates	The Percent (%)
<i>blaAmpC</i>	(32)	(91.4%)
<i>bla<sub>ctx-m</sub></i>	(28)	(80%)
<i>bla<sub>Tem</sub></i>	(22)	(62.8%)
<i>bla<sub>SHv</sub></i>	(8)	(22%)



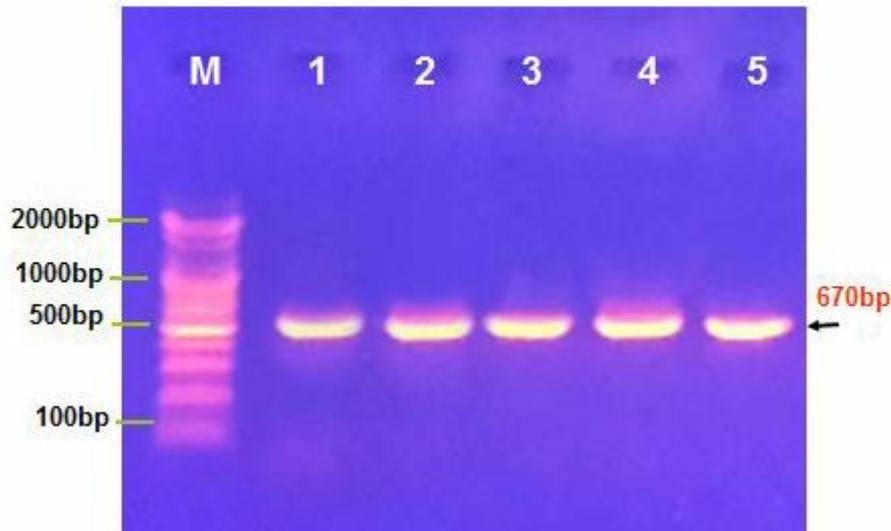
**Figure (1): Ethodium bromide-stained agarose gel electrophoresis to PCR amplified produced from of E.coli lance (1-5) isolates amplified with diagnostic *BLA<sub>CTX-M</sub>* gene show position at 247bp- electrophoresis performed at 80 volts for 1 hour.**



**Figure (2):** Ethodium bromide-stained agarose gel electrophoresis to PCR amplified produced from of E.coli lance (1-5) isolates amplified with diagnostic *bla<sub>SHV</sub>* gene show position at 410bp-electrophoresis performed at 80 volts for 1 hour.



**Figure (3):** Ethodium bromide-stained agarose gel electrophoresis to PCR amplified produced from of E.coli lance(1-5) isolates amplified with diagnostic *bla<sub>TEM</sub>* gene show position at 531bp- electrophoresis performed at 80 volts for 1 hour.



**Figure (4): Ethodium bromide-stained agarose gel electrophoresis to PCR amplified produced from of *E.coli* lance(1-5) isolates amplified with diagnostic *bla<sub>AMPC</sub>* gene show position at 670bp- electrophoresis performed at 80 volts for 1 hour.**

#### **Discussion:**

*Escherichia coli* are most common bacteria caused diarrhea in cattle and calves, and producing (extended spectrum beta-lactamases) or called (ESBLs) by this mechanism resistant for all groups of (beta-lactam antibiotics) (20).

ESBL-*E.coli* microorganisms are spread in all the world, with prevalence different from country to country and even in one country, and different from institution to another institution, based on several factors like the weather, the contamination level, virulence of the strains (21).

The current study aimed to determination the percentages of some extended spectrum beta-lactamases genes in *E.coli* isolated from diarrhea of cattle this percentage was (70%).

The results of (22) refer to (544) healthy adult worker that works in the factory was (75.5%), he refer to (77.3%) of *E.coli* isolated from (30) workers act in the animal farm were positive.

This study has an agreement with (23) who take (84) total isolates and detect the (55) isolates ESBL-*E.coli*; the prevalence was CTX-M gene; SHV gene and TEM gene (96.4%)(2.4%)(1.2%) respectively.

High contamination level with ESBL Enterobacteriaceae, up to (93.3%), are reported this supported by (24-26).

While our results different with (27)and (28) where the first reported the prevalence of (30%) of *E. coli* isolates. also different with (28) where *Escherichia coli* that isolated from (0.3 to 2.2%) of fecal samples in the United States, the United Kingdom,, and there are big gap with (29) where prevalence of *E.coli* was (14.6%) The study result also different with (30) and (31), where it was (27.3%) of isolates.

The genes were detected in a study; the prevalence of ESBL *E. coli* isolates was contract and depends on many factors like the area of the world and in different hospitals in the country.

In Iran was reported as (30.5%) also confirm in some countries e.g. turkey (17%), Korea (9.2%), India (27%), Lebanon (13.3%), (32) and (33).

The genes that detected in this study the prevalence of studies gene among isolate *E. coli* was a different value of the percentage of *bla<sub>Ampc</sub>* gene was (91.4%), *bla<sub>ctx-m</sub>* gene was (80%), *bla<sub>TEM</sub>* was (62.8%) and, finally, *bla<sub>SHV</sub>* gene was (62.8%) . there are several research and reports may have same, different and close to our outcome.

The disagreement with (34) found *bla<sub>TEM</sub>* gene; *blatem* gene; *bla<sub>ctx-m</sub>* gene were (9%) (33%) (9%) respectively.

There are different results with our report in where (33) confirm *E. coli* were ESBL- strain was (30.5%), The *TEM* gene; *SHV* gene *CTX* gene were (49%) (44%), (28%) respectively; according to the study the *TEM*-gene was more prevalent (33).

Also (35) has different results with our results, however, *bla<sub>CTXM</sub>* was (7.1%) and *bla<sub>TEM</sub>* was (18.7%) but *bla<sub>SHV</sub>* was (16.4%) that close for our result.

(36) Reported (52%) of isolates were detected as extended spectrum  $\beta$ -lactamases (ESBLs) by use PCR, all *E. coli* isolates possessed one or more ESBL gene. *CTX-M* type ESBL was the most dominant ESBL (87.2%) among the isolates. while those for *TEM*-type and *SHV*-type were (54.5%) and (21.8%) respectively.

Our result similar to the results of (37) wherever, ESBLs were (63.6%) of *E. coli*; ESBL genes was (57.3%); *bla<sub>CTX-M</sub>* was the commonest was (85.4%); *bla<sub>TEM</sub>* was (54.9%) and *bla<sub>SHV</sub>* was (32.9%) ; the results of ESBL genotyping has shown; The *TEM* gene was (49%); *SHV* (44%) gene, *CTX* (28%) genes (20).

Also very similarity with (38) ESBLs were detected (63.6%) and considered close to ours ; Other reports in India had found high percentage of ESBL strains about (41%) to (63.6%) in *E. coli*; ESBL- *Enterobacteriaceae* about from (5%) to (52%) and in some Asian countries about (10%) to (46.5%); while *bla<sub>CTX-M</sub>* gene was (75.2%) all that likely to ours (38).

The variations among studies were high resistance to other non $\beta$ -lactam antibiotics caused by treatment failure by these drugs (38) ; systemic inflammation; overuse of broad-spectrum antibiotics, play great important role for spread resistance phenomena in the *Enterobacteriaceae* family .; using some of antibiotics like trimethoprim-sulphamethoxazole and cephalosporins were associated with infections cases by ESBL strains also the animals which take contaminated ventilator for long use that will developed and spreading the ESBL organism (38) and (39) .

(40) Found percent of *E. coli* was (65%); *bla<sub>TEM</sub>* was (21.7%); *bla<sub>SHV</sub>* was (34%) and *bla<sub>CTXM</sub>* was (43.4%).

Percentage of antimicrobial drugs resistant *E. coli* in animals depend on many of the factors like size of herd, nature of calf housing, type of milking system, and nature of geographic location where the animals live; Treatment by broad spectrum antibiotics for prolonged time will be increased the spreading of resistant *E. coli* ; take milk contaminated by waste that contain antibiotics, but depending on colostrums from also in the cows that treated with antibiotics at drying off period had affected

negative on antibiotic resistance *E. coli* ; while Feeding healthy colostrums or healthy milk will be decreased the cases that resist the beta-lactam (41) .

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التحديد الجزيئي للجينات المقاومة للمضادات الحيوية البيتا لاكتام الواسعة النطاق الموجودة في الاشيريشيا القولونية المعزولة من الاسهال في الابقار  
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الكلمات المفتاحية: الاشيريشيا القولونية , الاسهال , جينات المقاومة للمضادات الحيوية , البيتا لاكتام

الخلاصة :

عدم الاستجابة للعلاج بالمضادات الحيوية يدعى المقاومة للمضادات الحيوية التي تتسبب ببعض المورثات المقاومة للمضادات الحيوية , هذه الظاهرة واسطة الانتشار في معظم انواع الجرثيم . مثل الاشيريشيا القولونية . ان مختصر (اي اس بي اي اس ) هو مثال نموذجي لذلك , دراسة بعض المورثات المسؤولة عن مقاومة المضادات الحيوية هو موضوع هذا البحث .

اخذت خمسين عينة من براز الابقار مصابة بالاسهال وزرعت على اوساط زرع عامية وانتخابية ثم استخلصت المادة الوراثية من الجرثيم (الدنا) , ومن ثم اجريت لها عملية فحص تفاعل السلسلة المتعدد لتقرأ النتائج تحت جهاز الاشعة فوق البنفسجية بعد ترحيلها بواسطة جهاز الترحيل الكهربائي .  
وجدت هذا الدراسة نسب بعض المورثات المقاومة للمضادات الحيوية البيتا لاكتام وه (بي ال اي ام بي سي ) كانت (91.4% ) , و المورثة ( بي ال اي سي تي اكس - ام ) كانت 80% , والمورثة ( بي ال اي تي اي ام ) كانت 62.8% واخيرا كانت نسبة المورثة ( بي ال اي اس اج في ) كانت 22% .

ان المورثة (بي ال اي ام بي سي ) هي الاكثر انتشارا , بينما الاقل انتشارا هي مورثة ( بي ال اي اس اج في ) .